Additional file 13: External validation process results. Eighty-seven TN patients of GSE21653 cohort were used for external validation. The same global process, as described for our cohort, was used and is described thereafter.

Clinicopathologic characteristics

We have selected 87 breast carcinomas that were shown to lack IHC expression of ER, PR and HER2. Contrary to our cohort, no significant age difference ($P = 0.26$) was found between the three clusters, named: C1', C2' and C3'. Histological grades were higher in C2 and C3 than in C1 ($P = 0.0031$). EFS did not show outcome difference according to cluster membership ($P = 0.22$) (Additional file 4). When GSE21653 was pooled with our 107 TN cohort, EFS results confirmed the trend found for our cohort ($P = 0.01$) (Additional file 4).

Gene-expression signatures

GES subtyping results are displayed in Figure 5 and Additional Table1.

Additional Table 1: GSE21653 gene-expression profiling in function of the three clusters.

GES name	Subtypes	Cluster 1' (*n* = 27)	Cluster 2' (*n* = 28)	Cluster 3' (*n* = 32)	*P*-value
Sorlie's SSP	Basal-like	0	25	24	< 0.0001
	HER2-E	4	1	1	
	Luminal A	1	0	0	
	Luminal B	6	1	4	
	NBL	2	0	0	
	Unclassified	14	1	3	
Hu's SSP	Basal-like	0	25	27	< 0.0001
	HER2-E	3	0	1	
	Luminal A	12	0	0	
	Luminal B	1	0	0	
	NBL	2	2	1	
	Unclassified	9	1	3	
Parker's SSP = PAM50	Basal-like	0	27	30	< 0.0001
	HER2-E	7	0	0	
	Luminal A	7	0	0	
	Luminal B	6	0	1	
	NBL	4	1	1	
	Unclassified	3	0	0	
Proliferation score (mean[sd])		8.80 [1.13]	10.15 [0.69]	9.82 [0.68]	< 0.0001
TNBCtype	BL1	0	11	4	< 0.0001
	BL2	1	3	5	
	IM	1	0	18	
	LAR	17	0	0	
	M	1	7	0	
	MSL	5	2	3	
	Unclassified	2	5	2	
Teschendorff's GES	CC+	0	5	0	< 0.0001
	CC+/IR+	2	22	31	
	ECM+	6	0	1	
	IR+	1	0	0	
	SR+	13	0	0	
	Unclassified	5	1	0	
VEGF profile (mean[sd])		9.18 [0.42]	10.03 [0.56]	9.72 [0.60]	< 0.0001
Glycolysis profile (mean[sd])		11.81 [0.46]	12.18 [0.77]	12.08 [0.52]	0.0637
Claudin-low	Claudin-low	0	0	7	0.0009
	Other	27	28	25	

SSP, single sample predictor; GES, gene-expression signature

Single sample predictor annotation

These signatures showed that C1' essentially contained non basal-like subtypes. This cluster was mostly composed of luminal A and B subtypes, and unclassified tumours. In C1', PAM50 subtyping identified 48% of luminal subtypes (luminal A [26%] and B [22%]), no basal-like and 26% of HER2-E. This last subtype was not found in our cohort. C2' was an almost pure basal-like cluster whatever the SSP used. In C2', one patient was subtyped as normal breast-like by means of PAM50 and Hu's SSP and basal-like by Sorlie's SSP. GSE21653 bio-clinical data only indicated that histology of this tumour was different from infiltrating ductal carcinoma. C3' included mostly basal-like subtypes, but to a lesser extent than C2' (94% [PAM50 SSP], 84% [Hu's SSP] and 75% [Sorlie's SSP]); these results are comparable with those obtained for our cohort.

Proliferation score

Proliferation score was significantly lower in C1' compared to C2' ($P < 0.0001$) and C3' ($P < 0.0001$). No difference was found between C2' and C3' ($P = 0.29$).

TNBCtype

TNBCtype classification assigned a TNBC subtype to 90% of TN GSE21653 tumours. C1' was LAR-enriched (68% of classified patients) and LAR subtypes were exclusively assigned to this cluster. This result confirmed that C1' was not a basal-like cluster as shown with SSPs, and our results (100% of LAR in C1 and 61.5% of classified patients). C2' was BL1- and M-enriched (47.8% and 30%, respectively). BL1 and BL2 represented 61% of C2'. C3' was IM- and BL1/BL2-enriched (60% and 30%, respectively). MSL, characterized by low expression of proliferation genes compared to M, was mostly found in C1' (20%). Except one, all IM subtypes were included in C3'. Immune response distinguished C3' from C2'. These results are comparable with those found for our cohort.

Teschendorff's GES

Steroid hormone receptor (SR) subtypes were exclusively observed in C1'. CC+/IR+ were almost exclusively included in C2' (78%) and C3' (97%). This GES confirmed TNBCtype subtyping of C1': SR and LAR, respectively. Immune response was almost exclusively assigned to C2' and C3' and did not separate these two clusters. These results are comparable with those found for our cohort.

VEGF profile

This 13-GES showed that angiogenesis varied in function of the three clusters ($P < 0.0001$). Angiogenesis score was significantly lower in C1' compared to C2' ($P < 0.0001$) and C3' ($P < 0.0006$). A trend was found between C2' and C3', with C2' > C3'.

Glycolysis profile

Glycolysis score showed a trend according to clusters ($P = 0.0637$).

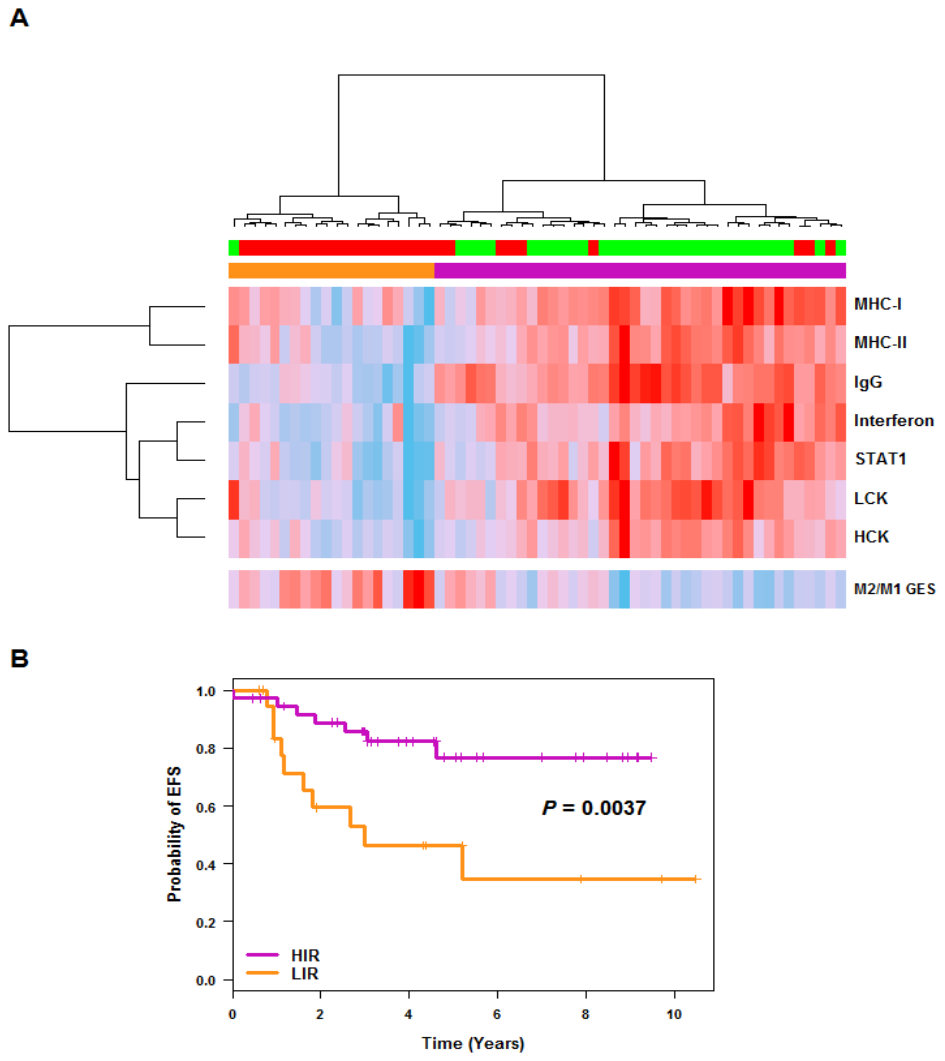
Claudin-low

Seven patients were subtyped as “claudin-low” (8%). The distribution was as follows: 0% in C1’ and C2’ and 22% (7/32) in C3’. So, C3’ was claudin-low-enriched but this subtype only represented around a fifth of its number. This result is comparable with the one found for our cohort (26% of C3).

Rody’s GES

For each immune module, metagene expressions were always significantly higher in C3’ compared to C2’ (data not shown). These results demonstrated that immune response could be considered as a hallmark of C3’. Ward’s hierarchical clustering method applied to these immune modules separated C2’ and C3’ patients in two clusters: one mostly composed of C2’ patients (95%) with low immune response (LIR) and the other composed of a majority (77.5%) of C3’ patients with high immune response (HIR) (Additional Figure 1). These results are comparable with those found for our cohort.

Considering C2’ and C3’ patients, EFS analyses were performed in function of Rody’s metagene subtypes. High expression of the majority of these metagenes was associated with a better outcome: MHC-II (P = 0.0272, HR = 0.61), IgG (P = 0.0024, HR = 0.70), Interferon (P = 0.0221, HR = 0.66), STAT1 (P = 0.0136, HR = 0.65), LCK (P = 0.0016, HR = 0.50). A trend was found for HCK (P = 0.08). MHC-I module was not associated with disease evolution (P = 0.26).



Additional Figure 1: C2'/C3' immune response dissection. (A) Ward's hierarchical clustering and heatmap showing the segregation of C2' (red) and C3' (green) patients in function of the seven Rody's metagenes (B lymphocytes [IgG]; macrophages and monocyte/myeloid lineage cells [HCK]; professional antigen-presenting cells [MHC-II]; T-cell [LCK]; cell types for presentation of intracellular antigens [MHC-I]; interferon signal transduction [STAT1]; interferon response [Interferon]) and M2/M1 GES: continuous colour scale, from minimum [deepskyblue] to maximum [red]. **(B)** Kaplan-Meier curves for event-free survival analysis of breast cancer patients with high immune response (HIR) and low immune response (LIR).

M2/M1 GES

Considering clusters C2' and C3', M2/M1 GES was significantly associated with bad outcome (EFS, $P = 0.03$), M2 protumorigenic macrophage genes were more expressed in C2' ($P < 0.0001$), which was characterized by LIR and bad outcome, and M1 tumour suppressor macrophage genes were more expressed in C3', which was characterized by HIR and better outcome. Of note, we did not find any correlation between VEGF score and M2 macrophage signature ($r = -0.10$; $P = 0.47$).

Single gene-expression intuitive approach

See Additional Table 2.

Additional Table 2: Expression level of chosen genes between the three fuzzy clusters of TN GSE21653 patients.

Genes	Characteristics	*P*-value	*P*-value C2' vs C1'	*P*-value C3' vs C1'	*P*-value C3' vs C2'	Results[a]	Attended results[a]	Validation[c]
AR	Luminal	< 0.0001	< 0.0001	< 0.0001	0.1120	1' > 2' = 3'	1' > 2' = 3'	Yes
ESR1		0.0883	-	-	-	-		No
PGR		0.0009	0.0015	0.0058	0.8438	1' > 2' = 3'		Yes
GATA3		< 0.0001	< 0.0001	< 0.0001	0.0550	1' > 2' = 3'		Yes
KRT18		< 0.0001	< 0.0001	< 0.0001	0.7206	1' > 2' = 3'		Yes
KRT19		0.0409	0.8345	0.1641	0.0425	2' > 3'		No
MUC1		0.0001	0.0021	0.0002	0.8331	1' > 2' = 3'		Yes
ERBB2	HER2-E	0.0023	0.0032	0.0133	0.8256	1' > 2' = 3'	1' > 2' = 3'	No[d]
CDH3	Basal-like	0.0107	0.0141	0.0387	0.8814	1' < 2' = 3'	1' < 2' = 3'	No[e]
EGFR		0.2536	-	-	-	-		No
KIT		0.0001	0.0003	0.0006	0.9600	1' < 2' = 3'		Yes
KRT5		0.0002	0.0004	0.0020	0.7980	1' < 2' = 3'		Yes
KRT6A		0.0009	0.0010	0.0128	0.6014	1' < 2' = 3'		Yes
KRT6B		< 0.0001	< 0.0001	< 0.0001	0.3050	1' < 2' = 3'		Yes
KRT14		0.0009	0.0007	0.00283	0.3644	1' < 2' = 3'		No[f]
KRT17		< 0.0001	< 0.0001	0.0014	0.0810	1' < 3' < 2'[b]		Yes[b]
CDH1	Epithelial cell-cell	0.5595	-	-	-	-	2' > 3'	No
CGN	adhesion	0.5568	-	-	-	-		Yes
CLDN3		0.9116	-	-	-	-		No
CLDN4	Claudin-low	0.8214	-	-	-	-		No
CLDN7		0.1915	-	-	-	-		No
EPCAM		0.0366	0.3939	0.4294	0.0276	2' > 3'		Yes
OCLN		0.0094	0.7008	0.0096	0.0774	1' > 3' and 2' > 3'[b]		Yes
MKI67	Proliferation	< 0.0001	< 0.0001	0.0002	0.3319	1' < 2' = 3'	1' < 2' = 3'	Yes
UBE2C		< 0.0001	< 0.0001	0.0001	0.8777	1' < 2' = 3'	or	Yes
AURKA		0.0002	0.0025	0.0003	0.8481	1' < 2' = 3'	1' < 3' < 2'	Yes
RACGAP1		0.0004	0.0003	0.0163	0.3590	1' < 2' = 3'		Yes
ABCA8	Breast stem cells	0.8521	-	-	-	-	2' < 3'	No
ALDH1A1		0.0006	0.0005	0.3290	0.0240	1' > 2' and 2' < 3'		Yes
CDH2	Epithelial-to-	0.8331	-	-	-	-	2' < 3'	No
FGF7	mesenchymal	0.3811	-	-	-	-		No
FOXC2	transition (EMT)	0.1171	-	-	-	-		No
SNAI1		0.7306	-	-	-	-		No
TGFB1	Extracellular matrix	0.0018	0.1166	0.2596	0.0011	2' < 3'		Yes
TWIST1		0.8465	-	-	-	-		No
VIM		0.0073	0.0068	0.0565	0.6421	1' < 2'		No
ZEB1		0.0009	0.0006	0.1877	0.0658	1' > 2' and 2' < 3'[b]		No
ITGA5	Cell migration	0.7877	-	-	-	-	2' < 3'	No
MSN		0.0004	0.0025	0.0010	0.9839	1' < 2' = 3'		No
CD4	Immune system	0.0015	0.0206	0.7387	< 0.0015	1' > 2' and 2' < 3'	2' < 3'	Yes
CD79A	response	< 0.0001	0.0307	< 0.0001	< 0.0001	2' < 1' < 3'		Yes
CXCL2		0.0391	0.4771	0.0304	0.3471	1' < 3'		No
IL6		< 0.0001	0.0041	< 0.0001	0.4132	1' < 2' and 1' < 3'		No
STAT1		< 0.0001	0.9712	< 0.0001	< 0.0001	1' < 3' and 2' < 3'		Yes
VAV1		< 0.0001	0.5396	0.0001	< 0.0001	1' < 3' and 2' < 3'		Yes

[a]: expression level between clusters
[b]: trend: 0.05 < p (Tukey) < 0.10
[c]: comparable expression in our cohort and in GSE21653
[d]: SSP subtyping identified more HER2-E tumours in C1' (GSE21653) contrary to C1 of our cohort (PAM50: 7 versus 1). This may explain *ERBB2* significant expression between C1' and C2'-C3'. In our cohort, we only identified a and its constant level between the clusters of our cohort.
[e]: In GSE21653, P-cadherin RNA expression is in accordance with current knowledge.
[f]: 1 < 2 in both studies; 1 < 2 = 3 in GSE21653

GO biological process terms enrichment

GO biological process terms enrichment demonstrated that C1' was characterized by digestion, steroid metabolic process, transport processes and oxidation-reduction process, C2' by tissue development and cell differentiation, and C3' by immune response (Additional Table 3). Comparing C2' to C1', C2' was enriched in mitotic cell cycle genes. These results are in agreement with those found for our cohort.

Additional Table 3: Biological process GO terms enrichment for validation TN GSE21653 patients.

	H1	H2	H3	C1 vs C2	C1 vs C3	C2 vs C3	C1 vs C2-C3
EASE	digestion	ectoderm development	immune response	M phase	immune response	immune response	M phase
	secretion	epidermis development	defense response	cell cycle phase	regulation of lymphocyte activation	lymphocyte activation	cell cycle phase
	cell-cell signaling	epithelial cell differentiation	inflammatory response	organelle fission	regulation of leukocyte activation	cell activation	organelle fission
	oxidation reduction	epithelium development	chemotaxis	mitotic cell cycle	regulation of T cell activation	leukocyte activation	nuclear division
	regulation of neurotransmitter levels	peripheral nervous system	taxis	nuclear division	regulation of cell activation	T cell activation	mitosis
	prostate gland development	development	locomotory behavior	mitosis	positive regulation of immune system process	positive regulation of immune system process	M phase of mitotic cell cycle
	steroid metabolic process	keratinocyte differentiation	response to wounding	cell cycle	leukocyte activation	regulation of lymphocyte activation	mitotic cell cycle
	cell-cell adhesion	epidermal cell differentiation	behavior	M phase of mitotic cell cycle	cell activation	regulation of cell activation	cell cycle process
	transmission of nerve impulse	cell motion	positive regulation of immune system process	cell cycle process	lymphocyte activation	regulation of leukocyte activation	cell cycle
	neuron differentiation	cell adhesion	cellular defense response	amine biosynthetic process	positive regulation of leukocyte activation	regulation of T cell activation	cell division
		biological adhesion					
EASE synthesis	digestion	tissue development	immune response	mitotic cell cycle	immune response	immune response	cell cycle
	secretion	cell differentiation					mitotic cell cycle
	oxidation reduction	cell motion					
	steroid metabolic process	cell adhesion					
ToppGene	digestion	epidermis development	immune response	mitotic nuclear division	immune response	immune response	nuclear division
	cell fate commitment	skin development	positive regulation of immune system process	organelle fission	regulation of immune system process	leukocyte activation	cell division
	lipid metabolic process	epithelial cell differentiation	regulation of immune system process	nuclear division	positive regulation of immune system process	lymphocyte activation	organelle fission
	steroid metabolic process	epithelium development	regulation of immune response	cell division	leukocyte activation	regulation of immune system process	mitotic nuclear division
	hormone metabolic process	keratinocyte differentiation	immune response-activating cell surface	mitotic cell cycle	lymphocyte activation	cell activation	mitotic cell cycle process
	organic anion transport	epidermal cell differentiation	receptor signaling pathway	chromosome segregation	regulation of lymphocyte activation	positive regulation of immune system process	mitotic cell cycle
	oxidation-reduction process	saliva secretion	positive regulation of immune response	mitotic cell cycle process	T cell activation	regulation of immune response	cell cycle
	anion transport	keratinization	defense response	cell cycle	regulation of immune response	T cell activation	chromosome segregation
	response to steroid hormone	neuroblast proliferation	immune response-activating signal	O-glycan processing	positive regulation of immune response	defense response	cell cycle process
	response to lipid	intermediate filament cytoskeleton organization	transduction	protein O-linked glycosylation	regulation of T cell activation	regulation of lymphocyte activation	negative regulation of nuclear division
			activation of immune response				
			lymphocyte activation				
ToppGene synthesis	digestion	tissue development	immune response	mitotic cell cycle	immune response	immune response	cell cycle
	steroid metabolic process	cell differentiation					mitotic cell cycle
	anion transport						
	oxidation-reduction process						
GOrilla	nitrogen compound transport	epidermis development	immune system process	organelle fission	immune system process	immune system process	nuclear division
	digestion	epithelium development	immune response	nuclear division	positive regulation of immune system process	regulation of immune system process	organelle fission
	single-organism transport	tissue development	positive regulation of immune system process	mitotic nuclear division	regulation of immune system process	positive regulation of immune system process	mitotic cell cycle process
	secretion	anatomical structure development	regulation of immune system process	single-organism process	positive regulation of immune response	regulation of immune response	mitotic nuclear division
	organic anion transport	epithelial cell differentiation	humoral immune response	mitotic cell cycle process	regulation of lymphocyte activation	immune response	cell cycle process
	ion transport	developmental process	defense response	cell cycle process	regulation of immune response	regulation of lymphocyte activation	mitotic cell cycle
	appendage morphogenesis	peripheral nervous system	regulation of immune response	O-glycan processing	immune response	defense response	single-organism process
	limb morphogenesis	development	positive regulation of immune response	protein O-linked glycosylation	regulation of T cell activation	regulation of leukocyte activation	cell cycle
	secretion by cell	keratinization	immune response-activating cell surface	microtubule polymerization or	regulation of leukocyte activation	regulation of cell activation	single-organism cellular process
	oxidation-reduction process	epidermal cell differentiation	receptor signaling pathway	depolymerization	immune response-activating signal	regulation of T cell activation	negative regulation of nuclear division
		keratinocyte differentiation	immune response-activating signal	single-organism cellular process	transduction		
			transduction				
GOrilla synthesis	transport and secretion processes	tissue development	immune response	mitotic cell cycle	immune response	immune response	cell cycle
	digestion	cell differentiation					mitotic cell cycle
	oxidation-reduction process						
Global synthesis	digestion	tissue development	immune response	mitotic cell cycle	immune response	immune response	cell cycle
	steroid metabolic process	cell differentiation					mitotic cell cycle
	transport processes						
	oxidation-reduction process						